Effects of Early Life and Adult Chronic Stress on Behavior and Physiology

Honors Thesis Research

Presented in partial fulfillment of the requirements for graduation "with honors research distinction in neuroscience" in the undergraduate colleges of the Ohio State University

by

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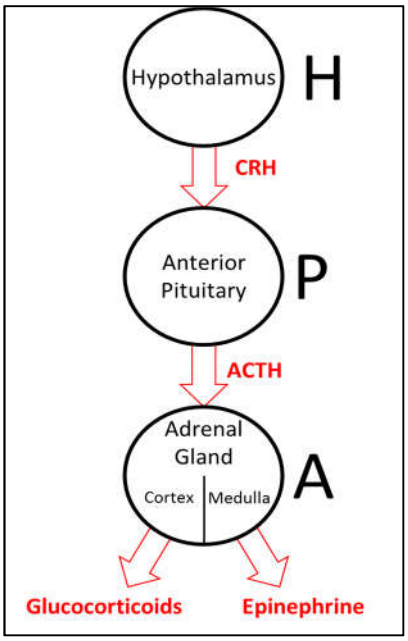
Abstract

In the modern and rapidly changing world, rates of perceived stress in humans have been increasing. Stress, which is caused by a disruption of homeostasis, activates the hypothalamic-pituitary-adrenal (HPA) axis, resulting in the release of glucocorticoids to make sufficient resources available for the body to respond to the stressor. Whereas glucocorticoids are beneficial during the acute stress response, chronic stress causes dysregulation of the endocrine system in an age-specific manner, leading to altered glucocorticoid concentrations in the serum and receptor expression in the brain. The goal of this study was to determine the effect of early life and adult chronic stress as a model of the organizational-activational capacity of glucocorticoids. The organization-activation hypothesis states that steroid hormones organize brain circuitry by a bout of high concentrations in early life and activate these circuits by a second event later in development. According to this hypothesis, early life stress and adult stress should organize and activate the glucocorticoid system to produce alterations in behavior and gene expression. To test this hypothesis, animals were exposed to maternal deprivation stress as an early life stressor (ELS) and/or unpredictable chronic mild stress (UCMS) to create four groups: Control/Control, Control/UCMS, ELS/Control, and ELS/UCMS. Following UCMS, all groups underwent behavioral testing and then gene expression was assayed. UCMS mice showed increased depressive-like responses toward adult chronic stress in weight gain and the sucrose preference test. However, the ELS did not affect behavioral outcomes or response to UCMS in adulthood, and few conclusions about gene expression could be drawn from this experiment. Future testing must be conducted to determine whether glucocorticoids follow the organization-activation hypothesis.

Introduction

Over billions of years of evolution, stress responses have evolved to allow individuals to rapidly respond to challenges in their environment to survive and reproduce. A stressor is defined as any disruption in homeostasis (Selye, 1956). Detection of a stressor triggers a series of physiological responses – blood vessels contract, the immune system is suppressed, and glucose availability increases – allowing the body to re-establish its equilibrium.



The stress response is coordinated by the hypothalamic-pituitary-adrenal (HPA) axis, which increases endocrine activity in response to a stressor. When a stressor is perceived, the hypothalamus releases corticotropin-releasing hormone (CRH), which in turn activates the anterior pituitary gland. In response, the anterior pituitary gland releases adrenocorticotropic hormone (ACTH) into the bloodstream. When ACTH reaches the adrenal gland, it stimulates the release of epinephrine from the adrenal cortex and glucocorticoids from the adrenal medulla. Epinephrine activates the sympathetic nervous system, allowing an immediate response to the stimulus. The sympathetic nervous system is responsible for the "fight or flight" response, increasing heart rate, widening bronchial passages, and inducing perspiration. Meanwhile, glucocorticoids (cortisol in humans or corticosterone in mice) allow for a long-term response to stress by altering metabolism and energy availability to certain tissues and organs. Glucocorticoids act by binding glucocorticoid receptors, which are found in nearly every cell in the body, and play a role in diverse functions including glucose metabolism and neuronal development.

Although stress can be beneficial for survival, chronic exposure to stressors or prolonged release of glucocorticoids can permanently disrupt allostasis. Allostasis is the adaptive process through which homeostasis is maintained by constant, subtle changes in physiology. Disruption of allostasis by chronic stress can lead to long-term dysregulation of the HPA axis, resulting in inappropriate glucocorticoid release and maladaptive "fight or flight" responses (Karatsoreos et al, 2013). The diverse actions of glucocorticoids throughout the body result in many physiological consequences when glucocorticoid signaling is affected by stress. In the brain, chronic stress can alter glucocorticoid receptor expression and cell proliferation in the hippocampus, amygdala, and pre-frontal cortex (McEwen, 2004; Mizoguchi et al, 2003). These brain regions regulate cognition and behavior, and they have been identified as factors in stress-related disorders such as depression and anxiety (Lupien et al, 2009). In the body, chronic stress decreases immune function and increases glucose availability, potentially contributing to various diseases including type-2 diabetes (Novak et al, 2013) and autoimmune disorders (Stojanovich & Marisavljevich, 2008).

Early life shapes the development of physiological and behavioral systems. The brain can be "programmed" or organized by both epigenetic and organizational changes in early life that persist into adulthood. Disruption of the early life environment, by anything from radiation exposure to food shortage, has been correlated with improper neuronal development, resulting in an increased risk for both behavioral and physiological disease (Boersma et al, 2014). Brain development continues from conception to adolescence, and any adversity experienced during this developmental period can alter critical developmental processes in the brain, including proliferation, migration, differentiation, myelination, and apoptosis (Rice & Barone, 2000).

Chronic stress in early life is particularly damaging to development of the brain and endocrine systems. Chronic stress during early life results in "allostatic overload" where

glucocorticoids are constantly present at high concentrations in the bloodstream. This disruption of allostasis is characterized by decreased glucocorticoid receptor expression, increased epigenetic methylation of genes related to the glucocorticoid receptor, and modified development of the nervous, endocrine, and immune systems (Danese et al, 2012; Lupien et al, 2009). These effects result in a hyperactive stress response and maladaptive behaviors which persist into adulthood.

The effects of chronic stress during early life have been studied at various stages of brain development in mouse models. Prenatal exposure to chronic stress decreases glucocorticoid receptor expression in adult offspring (Barbazanges et al, 1996). Similarly, postnatal exposure to a chronic stressor decreases glucocorticoid receptor expression and increases glucocorticoid sensitivity in adulthood (Mirescu et al, 2004; Meaney et al, 1996). Positive maternal behavior, such as increased licking and grooming in a mouse model, can decrease stress reactivity brought about by chronic stress in early life (Liu et al, 1997). Early life stress can also alter the duration of the stress response; mice exposed to chronic stress in early life return to baseline CRH levels quicker than those exposed to an acute stressor in early life (Romeo et al, 2006). This adaptive response indicates a level of plasticity in the HPA axis, which can be influenced by chronic stress during early development to affect glucocorticoid signaling in adulthood.

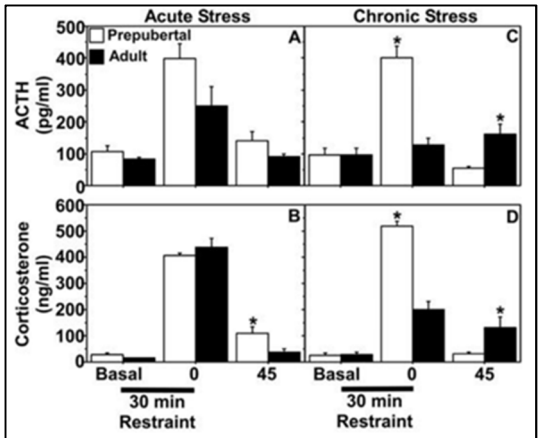


Figure obtained from Romeo et al, 2006

According to the organization-activation hypothesis, steroid hormones organize neural circuitry during early life and then can activate or modulate this circuitry in adulthood (Phoenix et al, 1959). Whereas effects of chronic stress on glucocorticoid signaling in early life and adulthood are well documented, the interaction between organization effects of early life stress and activation

effects of adult stress has not been extensively studied. A recent publication found that early life stress led to transcriptional changes in the ventral tegmental area (VTA) which predisposed mice to a depressive phenotype when experiencing adult stress (Pena et al, 2017); however, the social defeat stressors used in this experiment do not model realistic, environmental stressors.

This study predicted that early life stress alters responses toward chronic stress in adulthood. If true, then interactions between early life and adult stress would produce a novel phenotype in glucocorticoid expression and in behavior. Furthermore, this study evoked chronic stress responses using realistic models of daily stress encounters, allowing better translational conclusions to be drawn from this experiment. Understanding the interaction between early life and adult stress would allow for novel interventions in the treatment of psychological disease.

Methods

Animals: 16 pairs of adult male and female Smith-Webster mice were obtained from the Charles River and kept in polypropylene cages (30 x 15 x 14 cm) at an ambient temperature (22 ± 1°C) and standard lighting conditions (L:D 14:10 h ~130 lux/0 lux). Standard chow (Harlan 7912) and filtered tap water were available *ad libitum*. After one week of habituation, males and females were paired and allowed to mate over a one-week period. Males were then removed from the cage and euthanized. After birth, each litter was assigned either to an early life stress or standard, no-stress condition.

Early Life = no stress Adult = no stress N=15	Early Life = no stress Adult = stress N=16
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Maternal Deprivation: Early life stress was modeled using maternal deprivation on post-natal day 9 (PD9). Pups were separated from the dam and kept in a clean cage for 24 h as reported in Kember, et al (2012). During this time, cages containing the pups were kept in a separate room, maintained under a normal light cycle, and placed on a heating pad at 33±1°C to minimize external stressors. Dams were placed in a new cage and returned to the colony room. After 24 hours, pups and dams were returned to the original cage in the colony room. Maternal behavior was scored for three days before and after maternal deprivation to ensure that maternal care was not altered.

At PD21, offspring were weaned, and male offspring were group housed under standard conditions. Only males were used in this experiment due to significant sex differences in the stress response (Dalla et al, 2005; Mineur et al, 2006). At this time, mothers and female offspring were removed from the cage and euthanized, and maternal spleens were collected and stored at -80°C.

Unpredictable Chronic Mild Stress (UCMS): Males were further assigned to an adult stress or standard, no-stress condition, leading to a total of four experimental groups (N=15-16 per group). At 11 weeks of age, mice were single housed. The adult stress was administered one week after single housing. UCMS was used as a realistic model of everyday stress encounters (Willner et al, 1987; Mineur et al, 2006). The following stressors were applied on a random, daily schedule (Table 1) in the home cage: restraint stress (30 min in restraint tube), tilted cage (30° for 2 h), damp bedding (100 mL water added to bedding for 2 h), wet cage (100 mL water added to empty cage for 10 min) reversed light/dark (L/D) cycle, continuous lighting during

Table 1: UCMS Schedule

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
AM	Empty cage	Damp bedding		Wet cage		Continual light	
PM	Cage change		Restraint	Cage change	Cage tilt	Continual light	Empty cage
	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
AM	Wet cage	Restraint	Wet cage	Cage change		Reverse L/D	
PM	Cage change			Cage tilt	Empty cage	Reverse L/D	Damp bedding

the night phase (24 h lights on), empty cage (no bedding for 2 h), and unscheduled switching of cages. Following UCMS, behavior was tested and then tissues were collected.

Behavior Testing. Following UCMS, all animals underwent one week of testing to analyze depressive- and anxiety-like behavior. On nights 1-3, mice underwent a sucrose preference test. On night 5, mice were tested in the open field, elevated plus maze, and forced swim task. Then, on night 7, the animals were euthanized by rapid decapitation, and their tissues were collected.

Sucrose preference was used as an analysis of depressive-like behavior by measuring consumption of a 2% sucrose solution (Fonken & Nelson, 2013). To control for the novelty of the bottle, mice were first administered water in modified bottles for 5 h starting at the onset of the dark phase on night 1. On the 2nd and 3rd nights, mice were given a choice of water and the 2% sucrose solution in the modified bottles for 5 h. The change in mass of the solutions was recorded at the end of each session. Bottle placement was randomly assigned and counterbalanced to control for side preference. Sucrose preference was measured as the percentage of sucrose solution to total liquid (sucrose solution and water) consumed during the experimental period.

Performance in the open field was used to test anxiety-like behavior. The open field consists of a light- and sound- controlled acrylic chamber (40 x 40 x 40 cm) surrounded by stacked infrared beam emitter/detectors (San Diego Instruments, Inc., San Diego, CA) to distinguish vertical and horizontal movement. Mice were placed in the chamber, and their locomotor activity was measured for 20 min using Photobeam Activity System Software (San Diego Instruments, Inc., San Diego, CA). Interruptions to the infrared light source were recorded, and results were analyzed for total activity, amount of activity in the center of the open field, and number of rears to generate a mean central tendency statistic.

The elevated plus maze was used to test anxiety-like behavior. The maze consists of two exposed (67 x 5.5 cm) and two enclosed (67 x 5.5 cm, 15 cm tall) arms. Mice were placed in the center of the platform, and their behavior was videotaped for 5 min. Recordings were analyzed by an observer unaware of the experimental conditions for latency to enter open arm, number of visits to open arms and total time spent in open arms.

Lastly, a five-minute forced swim test was used to assess depressive-like behavior. Mice were individually placed into a 5000-mL beaker containing approximately 2000-mL of water at 24±1°C for five minutes. Their performance was videotaped. After completing the task, mice were dried and returned to their cage. Between each trial, the beaker was drained and cleaned. Recordings were analyzed by an observer unaware of the experimental conditions for latency to float, number of float bouts, float time, and climbing behavior.

Tissue Collection. Following behavioral testing, mice were euthanized by rapid decapitation. Spleens and adrenal gland were collected, weighed, and then stored at -80°C. Brains were placed in RNAlater and stored at 4°C. Hippocampi and hypothalamuses were later dissected from brains and stored at -80°C. Spleen mass was used as a preliminary measure of interaction between stress and the immune system. Adrenal mass was used as a preliminary measure of HPA-axis function.

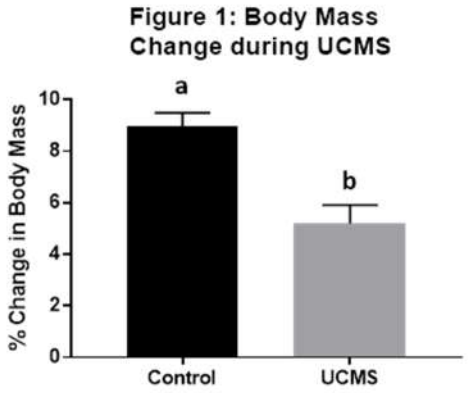
PCR: RNA was extracted from homogenized tissues (Ultra-Turrax T8; IKA Works, Wilmington, NC) using RNAzol reagent (QIAGEN, Austin, TX) according to the manufacturer's instructions. RNA pellets were then resuspended in 30 μL of ultra-pure water, and RNA was quantified using a spectrophotometer (NanoDrop, Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using M-MLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA) and diluted

to a concentration of 40 ng of cDNA per sample for subsequent PCR. Finally, gene expression for glucocorticoid receptors (GR) and brain-derived neurotrophic factor (BDNF) was assayed. Taqman Fast advanced master mix was used in a 20 μL multiplex reaction with predesigned probes for BDNF and GR (Life Technologies). Expression of these targets was normalized to an endogenous 18s-rRNA signal and quantified using a serially diluted standard curve of pooled cDNA. The following cycling conditions were used in a 2-step real time PCR reaction: 95°C for 20 s, 40 cycles of 95°C for 3 s, and then 60°C for 30 s. GR is a direct measure of glucocorticoid activity in the brain, and BDNF is important in neural proliferation and used as an indicator of neural atrophy resulting from high concentrations of glucocorticoids in the brain.

Data Analysis. All comparisons between groups were tested for equal variance, normal distribution, and extreme values. Values that exceeded Grubb's test statistic for outliers were excluded. Normally distributed data was analyzed using a one-way ANOVA with early life and adult stress as between subject factors, and post hoc tests of were performed using Tukey's HSD. Nonparametric data was analyzed using the Kruskal-Wallace H-test with early life and adult stress as between subject factors, and post hoc tests of significant interactions were performed using Dunn's test. All models of variance included litter as a co-factor to control for litter effects. Analyses were completed with SPSS v24 software and then plotted using GraphPad Prism 7. A significance level of $p \leq 0.05$ was used for all analyses.

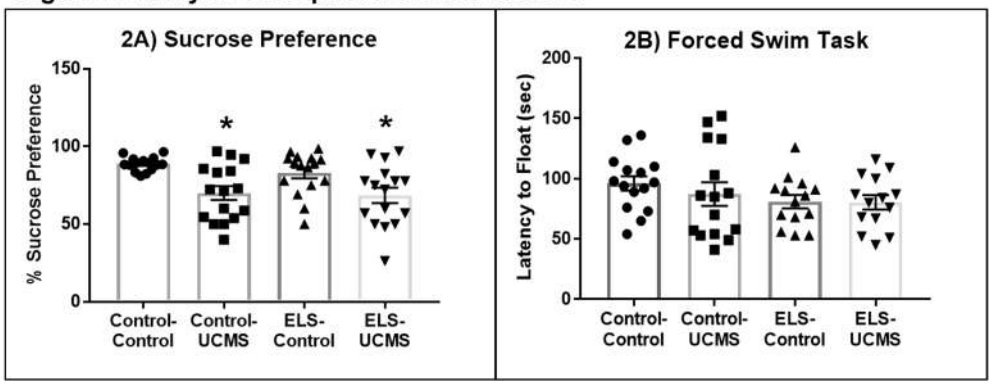
Results

Body mass gain during UCMS. Body mass was measured before and after the UCMS stressor, and percentage of body mass gained during this period was calculated. UCMS significantly decreased the percent body mass gained during this period relative to the control ($F_{1,61} = 15.073$, $p < 0.001$, see Fig 1). Tukey's post-hoc test revealed significant differences between Control-Control and ELS-UCMS groups ($p = 0.008$) as well as between ELS-Control and ELS-UCMS groups ($p = 0.003$). Decreased weight gain is a common anhedonia-like response from UCMS (Pothion et al, 2004) and provides evidence that this model was successful.



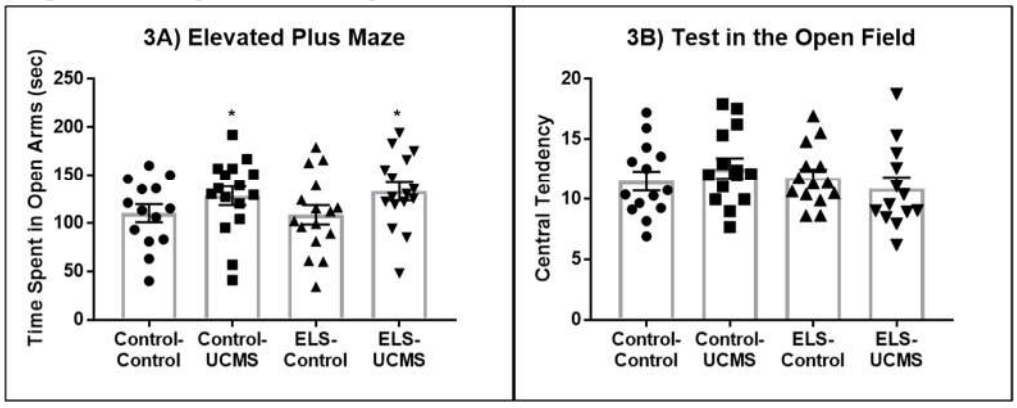
Depressive-like Behavior. In the sucrose preference test, UCMS decreased sucrose preference regardless of early life condition when compared to controls. In the absence of an adult stressor, mice exposed to ELS exhibited an intermediate phenotype relative to both controls and UCMS mice. There was a significant effect of condition on sucrose preference (Kruskall-Wallis non-parametric test: $\chi_{3,63} = 13.440$, $p = 0.004$, Fig 2A). Dunn's post-hoc test for non-parametric data indicated that the Control-Control group had significantly higher sucrose preference than the Control-UCMS ($p = 0.02$) and ELS-UCMS ($p = 0.02$) groups. In the forced swim task, there were no significant differences between groups ($p > 0.05$, Fig 2B).

Figure 2: Analysis of Depressive-like Behavior



Anxiety-like Behavior. In the elevated plus maze, UCMS increased the amount of time spent in the open arms in both control and ELS mice, indicating a decreased anxiety-like response. There was a significant effect of adult stress on time spent in the open arm ($F_{1,62} = 4.788$, $p = 0.03$, Fig 3A). Tukey's post-hoc test indicated no further interactions between groups (all $p > 0.05$). In the open field, no significant effects of condition were found for central tendency ($p > 0.05$, Fig 3B).

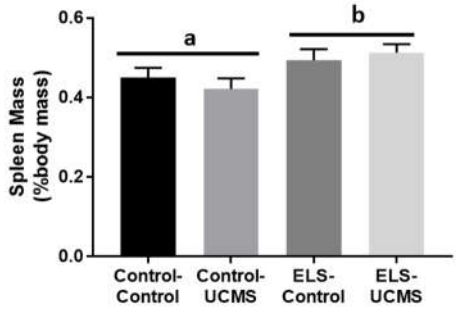
Figure 3: Analysis of Anxiety-like Behavior



Tissue masses. Early life stress had a significant effect on spleen mass; the spleens from mice exposed to early life stress were a greater percentage of total body weight than in mice that did not experience early life stress ($F_{1,62} = 7.428$, $p = 0.009$, Fig 4). A statistically significant litter effect was also observed ($F_{1,62} = 6.614$, $p = 0.01$), but further analysis confirmed that there was no correlation between maternal and offspring spleen mass (Spearman's $\rho = -0.063$, $p > 0.05$). There

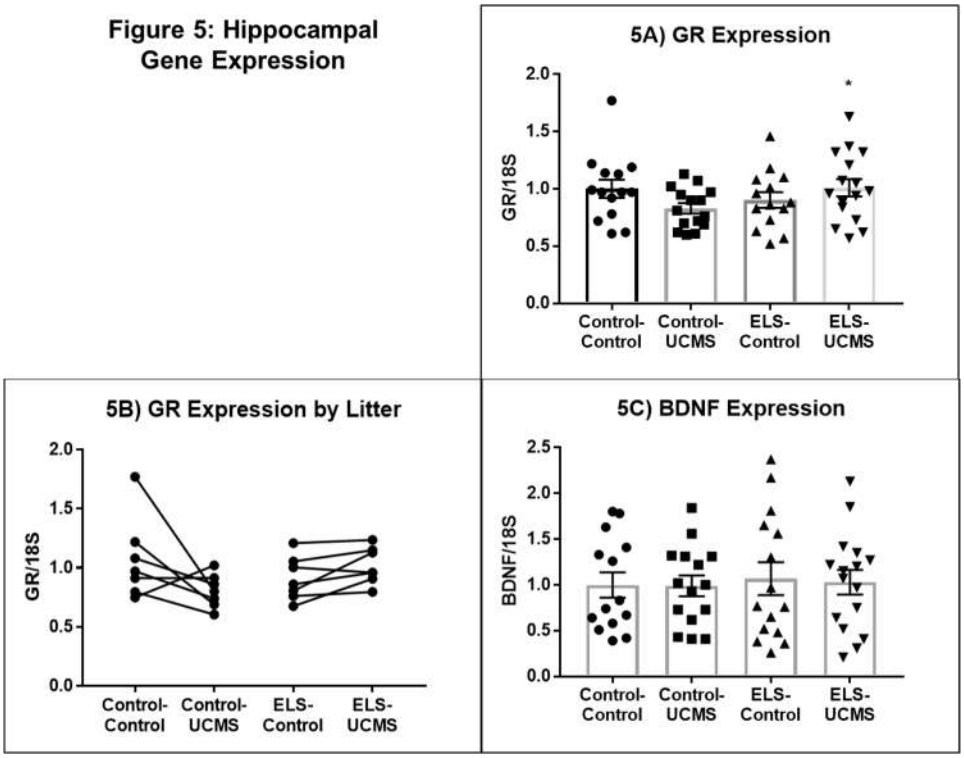
was no significant effect of condition on adrenal mass ($p > 0.05$). It should be noted that several adrenal samples were torn during necropsy and could not be weighed, so the power of this analysis was reduced ($N = 50$).

Figure 4: Tissue Collection Spleen Mass



qPCR for GR and BDNF. To analyze the role of glucocorticoids in the observed behavior, glucocorticoid receptor (GR) and brain-derived neurotrophic factor (BDNF) expression in the hippocampus were analyzed using quantitative PCR (qPCR). mRNA expression ratios were created for GR:18S and BDNF:18S to compare relative gene expression. In the GR data set, one extreme value was removed ($G = 4.13 > G_{max}$, $G_{max} = 3.023$ for $N=59$ and $\alpha=0.05$). There was a significant interaction between early life and adult stress ($F_{1,59} = 5.565$, $p = 0.022$, Fig 5A), but no effects of early life or adult stress were seen in isolation. There was also a significant effect of litter in the model ($F_{1,59} = 5.916$, $p = 0.018$); therefore, a plot of litter interaction was also provided (Fig 5B). In this plot, each litter is represented by one line that shows the difference in GR expression between mice exposed to adult stress and those who experienced controlled conditions. The litters are further separated into those which experienced early life chronic stress (on right) and those who did not (on left). Except for one litter, mice that experienced no stress in early life decreased GR expression when exposed to adult stress, while mice that experienced early life chronic stress increased GR expression when exposed to adult stress. This indicates a level of plasticity in the stress response. There was no effect of condition in the expression of BDNF ($p > 0.05$, Fig 5C).

Figure 5: Hippocampal Gene Expression



Discussion

UCMS altered anxiety- and depressive-like behavior in adulthood. UCMS mice exhibited less anxiety-like behavior in the elevated plus maze and more depressive-like behavior in the sucrose preference test than adult controls. No behavioral differences were observed between early life stress (ELS) and early life control mice. ELS increased spleen mass as a percentage of total body weight, and ELS also altered glucocorticoid receptor expression in conjunction with UCMS relative to adult control groups. These results provide weak evidence that UCMS was successful as a chronic stressor, but they do not indicate that the maternal deprivation model of ELS was successful in producing a stress response. Therefore, the null hypothesis cannot be rejected.

The effects of the UCMS stressor provide evidence of an adult stress response. In addition to the aforementioned depressive-like behavior in the sucrose preference test, UCMS mice gained

less weight than adult stress control groups in the duration of the stressor. These measures of UCMS confirm previous knowledge of chronic adult stress. The decreased anxiety-like response in the elevated plus maze contrasts previous models of chronic stress, which often exhibit a co-morbidity between depressive- and anxiety-like behavior, but these data likely result from UCMS as the model of adult stress. Previous experiments have reported that UCMS can induce depression-like phenotypes without increasing anxiety-like behavior in many strains of mice (Mineur et al, 2006). No interactions between UCMS and ELS were observed in the behavioral assays. This suggests that the organization-activation hypothesis may not apply to glucocorticoid hormones or that the maternal deprivation model of ELS was unsuccessful.

In response to chronic stress in adulthood, glucocorticoid receptor (GR) expression in the hippocampus decreases as receptors down-regulate in response to constant, high glucocorticoid concentrations (McEwen, 2004). GR expression showed a downward trend in mice exposed to either ELS and UCMS, but not both. When mice received both ELS and UCMS, there was a significant interaction between these conditions, restoring GR expression to control levels. A similar result has been seen in avian models, where animals born in a stressful environment can adapt better to stressful environments in adulthood by altering epigenetic methylation of the GR promoter gene (Rubenstein et al, 2016). This result suggests that early life chronic stress might adapt an animal to a high stress environment in adulthood. The BDNF data do not corroborate this hypothesis of stress adaptation, as BDNF showed no significant effects of ELS or UCMS. Under this stress adaptation hypothesis, BDNF concentrations would be reduced in the ELS-Control and Control-UCMS groups and then restored to baseline with ELS and UCMS. Therefore, these data should be confirmed in future studies.

ELS via maternal deprivation affected offspring, altering spleen mass and interacting with UCMS to alter glucocorticoid receptor expression. However, these results do not appear to be consistent with previous results of the early life, chronic stress response. Early life chronic stress is characterized by increased reactivity to stress and resting-state glucocorticoid concentrations (Gutman & Nemeroff, 2002). Furthermore, increased spleen mass in the ELS group relative to the control indicates that maternal deprivation might have increased immune activity, as increased spleen mass is correlated with an increased capability to fight disease. This result is not supported by the literature, as chronic, high concentrations of glucocorticoids have been found to suppress immune function (Maccari et al, 2014). These results provide evidence that the maternal deprivation model of ELS used in this experiment did not produce the expected stress response.

Maternal separation models have been disputed in recent years (Tan et al, 2017), and these data add to the evidence that these models of ELS are insufficient to produce a behavioral stress response in mice. Maternal separation is characterized by several bouts of maternal care interruptions lasting 3-4 h/day, and maternal deprivation involves a single, long interruption in maternal care typically lasting 24 hours (Pryce et al, 2005). While there is extensive literature supporting the use of these models in rats, there are few successful experiments exhibiting maternal separation or maternal deprivation in mice. This does not consider reporting bias, as failed experiments are less likely to be published and provide evidence against this method. Thus, future research should be conducted to confirm the validity of this model as an early life stressor in mice.

Future Directions

This study investigated the organization and activation of the glucocorticoid system; however, the maternal deprivation model of ELS was not successful, and the UCMS model showed a contradictory result in the elevated plus maze. Therefore, this experiment should be repeated with a different model of early life stress or animal strain. Pena and colleagues (Pena et al, 2017) report a successful model of ELS for C57Bl6 mice in which maternal separation for 4 hr/day from post-natal days 10-17 was combined with limited bedding to induce an early life stress response. Additionally, Mineur reports that strain differences in the chronic stress model of UCMS may play a significant role in the observed stress response (Mineur et al, 2006). Lastly, extra care should be taken to conduct this experiment in a stress-free environment. Mice in this experiment were housed in static cages and left undisturbed; however, other experiments were being conducted in the colony room which may have acted as a stressor to the control animals, confounding results.

Due to the failure of the model for ELS, further analysis of the stress response was abandoned. Blood was collected at two time-points following the forced swim test, immediately after the swim (N=14-16 for each subgroup) and after 45 or 90 minutes (N=7-9). Blood was also collected at a third time-point, following tissue collection (N=14-16). Using these samples, we planned to generate a serum corticosterone response curve with data points at the resting state, following the stressor, and 45 and 90 minutes after the stressor for each condition. Corticosterone would be quantified using enzyme-linked immunoassay (ELISA). In response to chronic stress, the glucocorticoid response can change both temporally and in magnitude (as seen in Romeo et al, 2006); therefore, we hypothesize that a new phenotype will emerge when ELS and UCMS interact.

Further assays for gene expression were also abandoned, and they should be conducted in future experiments to provide a more complete analysis of the effects of chronic stress on gene

expression. It was planned to analyze expression of the dopamine D2 receptor, serotonin 5HT1A and 5HT2A receptors, and TRK-α receptor in the hippocampus, as well as for IL-1 and IL-6 cytokines in the spleen to further analyze interactions between the HPA axis and depressive and immune phenotypes. Furthermore, we planned to run bisulfite sequencing PCR for methylation of the glucocorticoid receptor promoter in the hippocampus, as previous studies have reported a significant epigenetic contribution to ELS phenotypes (Weaver et al, 2004).

Broader Implications

Rates of perceived stress in humans have been rapidly increasing; from 1983 to 2009, stress as measured by the Perceived Stress Scale has increased by approximately 20% across all demographics in the United States (Cohen et al, 2012). Chronic stress often results in anxiety and depression, conditions which together represent more than 50% of the burden from mental health disorders worldwide (Whiteford et al, 2015). Understanding the temporal organization of the glucocorticoid system in response to chronic stress will allow for novel methods for preventative care in the treatment of mental health disorders.

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